Cellular Alchemy and the Golden Age of Reprogramming

George Q. Daley[1,2,3,4,*]
[1]Stem Cell Transplantation Program, Division of Pediatric Hematology/Oncology, Manton Center for Orphan Disease Research, Howard Hughes Medical Institute, Children's Hospital Boston and Dana Farber Cancer Institute, Boston, MA 02115, USA
[2]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA
[3]Broad Institute, Boston, MA 02115, USA
[4]Harvard Stem Cell Institute, Boston, MA 02115, USA
*Correspondence: george.daley@childrens.harvard.edu
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The 2012 Nobel Prize in Medicine or Physiology recognizes the architects of two of the great paradigm-shifting discoveries of the last half-century of biology. In experiments performed nearly 50 years apart, Gurdon and Yamanaka made feasible the reawakening of pluripotency inherent in all cells and challenged forever our notions of cellular identity.

In a shared accolade that was widely anticipated, the Swedish Academy recognized Sir John Gurdon of the United Kingdom and Shinya Yamanaka of Japan for their demonstration that differentiated somatic cells can be reverted, or reprogrammed, to an embryonic state. Their momentous contributions have fundamentally shaped our understanding of cellular differentiation and dedifferentiation, ushered in powerful opportunities for modeling human disease in vitro, and brought personalized cellular and genetic therapies closer to reality. Their work has taught us to accept that cells are plastic and malleable and can be engineered as tools for research and novel medicines. Indeed, these two scientists achieved the seemingly impossible in cellular alchemy, transforming the leaden state of somatic tissues into the golden opportunity of pluripotent stem cells.

To understand the impact of the discoveries by Gurdon and Yamanaka, one must review a bit of scientific history to appreciate the intellectual traditions that have been so summarily altered. Ever since Leeuwenhoek trained his microscope on the earliest steps of embryogenesis, scientists have marveled at how a single-celled zygote can transmogrify into complex multicellular fates and organismal form. As one cell divides to become two, then two to become four, and so on, scientists have wondered at what point do the cells became unequal in their properties and in their destinies, and by what mechanisms? In 1885, August Weismann published the theory that development worked by "qualitative divisions" among daughter cells, which segregated subsets of heritable material to specify their unique traits. In 1888, Wilhelm Roux pricked and ablated one cell of a two-cell frog embryo and observed formation of a "half-embryo," suggesting that, even at the two-cell stage, the embryonic blastomeres were nonequivalent, an experiment consistent with the notion of qualitative division. In 1892, Hans Driesch challenged that interpretation when he microdissected and separated sea urchin embryos at the two-cell stage and observed formation of two equivalent sea urchins, thereby extending the notion of nuclear equivalence at least to the two-cell stage. Later, Hans Spemann tied tiny hairs from his daughter's head around early-stage newt embryos, separating early blastomeres and observing formation of two normal newts, albeit one smaller than the other, proving developmental equivalence up to the eight-cell stage. Spemann famously envisioned but never technically realized a "fantastical experiment" whereby the nucleus of a highly differentiated cell might be transplanted back to the egg to test whether it would remain specialized or would manifest embryonic potential. Reporting precisely that experiment in 1952, Briggs and King showed that, when nuclei were transplanted from the blastula stage cells of a frog embryo (*Rana pipiens*), a point when cellular specialization had already begun, a third of reconstructed zygotes produced a swimming tadpole (Briggs and King, 1952). Their subsequent nuclear transplantation studies documented that cells from the gastrula stage showed reduced success, and ultimately, nuclei of the endoderm of the tail bud stage generated only abnormal embryos, thus suggesting that cells lose the ability to support normal embryonic development as development and cell specialization progresses.

Working in the more pliable *Xenopus* system, Gurdon diligently performed thousands of nuclear transfers, confirming that the efficiency of generating developmentally normal frogs declined with increasing maturity of donor cells. Most importantly, however, he established that *normal* development to adulthood could be achieved by transfer of fully differentiated nuclei from intestinal cells of feeding-stage larvae (Gurdon, 1962; Gurdon and Uehlinger, 1966). These remarkable experiments established that at least some highly differentiated cells retained all relevant hereditary information as the zygote and that cell specialization need not entail the discarding of nuclear material during cell specialization nor any irreversible genetic alteration to the cell. Gurdon's profound contribution represents the foundation of our current assumptions about nuclear equivalence. His bold and painstaking experiments have stood the test of time and are the

intellectual foundation of the excitement that has consumed the last 15 years of stem cell biology.

Gurdon's successful nuclear transfer in the amphibian led to widespread attempts in other organisms to produce genetically identical animals through nuclear transplantation, a process dubbed "cloning." Such attempts were technically challenging due to the small size of the mammalian egg and its sensitivity to experimental manipulation. In 1983, McGrath and Solter reported successful development of a nuclear transplantation procedure in mice, allowing them to observe a >90% frequency of live births following transfer of a zygotic nucleus into an enucleated zygote, which proved their technical facility. However, when they performed nuclear transfer from cells at later stages, they observed progressively lower efficiency and failed in attempts at nuclear transplantation from the eight-cell stage and inner cell mass (McGrath and Solter, 1984). In a statement that dissuaded many from pursuing further attempts at cloning mammals, they concluded that "reprogramming after transfer into the zygote is impossible in the mammalian embryo" and that "the cloning of mammals by simple nuclear transfer is biologically impossible" (McGrath and Solter, 1984). Despite these admonitions, efforts continued, in part driven by the value to animal husbandry of producing identical herds of farm animals through cloning. In the late 1980s and early 1990s, nuclear transplantation of embryonic cells proved successful in cloning numerous mammals, including sheep, pigs, cows, mice, and monkeys. In 1996, Campbell and Wilmut succeeded in deriving two cloned sheep from a differentiated cell line established from a 9-day-old embryo (Campbell et al., 1996), and a year later they achieved worldwide acclaim by successfully deriving a single sheep, "Dolly," from the mammary cells of an adult ewe (Wilmut et al., 1997). In their case, success necessitated adaptations of the cell cycle of the donor nucleus to better match that of the recipient oocyte. These data extended Gurdon's principle of nuclear equivalence for all somatic cells to fully differentiated cells of an adult mammal, an experiment that had implications far beyond the scientific community.

A year after Dolly, the field was again rocked by the successful establishment in culture of human embryonic stem cells (hESCs) by Jamie Thomson and colleagues (Thomson et al., 1998). The juxtaposition of these two major advances suggested a compelling prospect: perhaps the two techniques could be melded together to isolate hESCs from blastocysts generated by nuclear transfer using donor nuclei from patients with specific genetic diseases. With success, such cells could be differentiated into tissues affected by that disease to illuminate disease mechanisms, or the gene defects in the cells could be repaired, generating normal hESCs and normal derivative tissues for transplantation. In a proof-of-principle experiment for just such a potential therapeutic application, Rudolf Jaenisch and I collaborated to derive ESCs by nuclear transfer from cells of mice with genetic immune deficiency (Rideout et al., 2002). Our teams corrected the responsible gene defect, differentiated the repaired cells in vitro into hematopoietic stem cells, and then subsequently engrafted mice. The resulting mice showed partial restoration of B and T cell lineages and immune function as measured by serum levels of immunoglobulin (Rideout et al., 2002). Lorenz Studer's group applied a similar strategy to demonstrate production of dopaminergic neurons from stem cells made from somatic cell nuclear transfer (ntES cells), followed by transplantation to relieve motor symptoms in a murine model of Parkinson's disease (Barberi et al., 2003). Studer's later experiments demonstrated also that the lack of an immune response of the input DA neurons contributed to their more robust function, establishing the value of generating rejection-proof, autologous therapeutic tissues from a patient's own cells (Tabar et al., 2008).

Though in principle, somatic cell nuclear transplantation (SCNT) offered a means of producing autologous tissues for research and transplantation, in practice, the procedures were technically cumbersome, labor intensive, and time consuming, reducing their practicality. Moreover, the approach raised the specter that scientists might try to clone human babies, which was never the intention of any credible practitioners of SCNT. Indeed, within the United States, the manipulation of the human embryo entailed by these procedures caused tremendous controversy. Funding of such research was prohibited by US law, and President George W. Bush extended funding restrictions to any newly created hESC lines, effectively precluding a role for the US National Institutes of Health in fostering research in this arena. Programs funded through private philanthropy, like the Harvard Stem Cell Institute, or through state government bonds, like the California Institute for Regenerative Medicine, sprung up to pursue the compelling scientific possibilities of this new field. Even with private or state funding, however, scientists garnered little access to human oocytes needed for reprogramming, and efforts to reprogram human nuclei in heterologous species like the cow and pig met with abject failure.

The legacy of Gurdon had established the principle of conservation of the genome during cellular differentiation. His success with nuclear transfer established that molecular machinery within the egg cytoplasm was sufficient to reprogram a somatic genome to a pluripotent state. These early experiments compelled scientists to envision strategies to purify and characterize biochemical activities from egg cytoplasm. Some hypothesized a role for pluripotency factors like Oct4 and Nanog in reprogramming, but all imagined the ultimate task to be daunting. In 2004, in a review entitled "The First Half-Century of Nuclear Transplantation," Gurdon wrote: "We believe that the remarkable reprogramming activity of egg and oocyte cytoplasm will eventually be understood in terms of identified molecules, and it may well be possible to apply the equivalent human molecules to reprogram somatic cells, which would have to be proliferated in vitro as are embryonic stem cells (Gurdon and Byrne, 2003)." The challenge seemed infinitely complex, and Gurdon surmised that "a second half-century of nuclear transplantation should identify the molecules and mechanisms that achieve nuclear reprogramming."

Given this daunting challenge, the experiments of Shinya Yamanaka are all the more remarkable. In the early years of the last decade, Yamanaka was

establishing a growing reputation in the stem cell community for his elucidations of the role of factors like E-Ras and Nanog in ESC pluripotency. Knowing that experimental fusion with hESCs effectively reprogrammed the somatic donor nucleus of a differentiated cell (Cowan et al., 2005), Yamanaka and his graduate student Kazutoshi Takahashi compiled a set of gene candidates that might account for the pluripotent nature of hESCs. Rather than endeavor to painstakingly purify the enzymatic complexes responsible for reprogramming by the egg cytoplasm, Yamanaka hypothesized that he might change the fate of somatic cells by ectopic expression of transcriptional regulators, DNA-binding proteins that had been shown in other systems to induce fate changes (e.g., MyoD; Davis et al., 1987). Among the many hundreds of candidates that distinguish ESCs from fibroblasts, Yamanaka winnowed the set to 24. Takahashi then introduced all 24 candidate factors at once into a culture of mouse embryonic fibroblasts that had been engineered to carry a reporter gene active in pluripotent stem cells. Remarkably, the experiment worked, yielding cells that appeared morphologically like ESCs, expressed many of the expected markers of ESCs, and showed the ability to differentiate into cells from all three germ layers in vitro and in chimeric mice. In a second inspired experimental strategy, Takahashi and Yamanaka repeated the experiments multiple times with subsets of factors, noting that failed experiments were most informative for identifying the essential core reprogramming factors. Ultimately, they whittled down the list to the now famous core Yamanaka factors: Oct4, Sox2, KLF4, and c-Myc, which are alone sufficient to revert a somatic cell to its latent embryonic potential. They dubbed their products "induced Pluripotent Stem Cells," now widely known as iPS cells (iPSCs) (Takahashi and Yamanaka, 2006; see also Document S1, available online, for Takahashi and Yamanaka [2006] annotated by Konrad Hochedlinger).

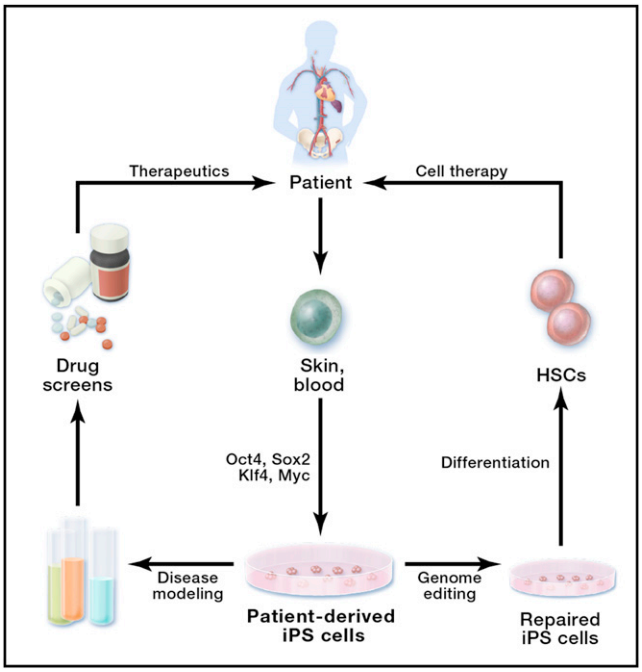

Figure 1. Reprogramming Allows Derivation of Patient-Specific Induced Pluripotent Stem Cells

Such cells provide a substrate for studies of disease mechanisms and pathogenesis; screening of small-molecule or protein therapeutics; and, through directed differentiation, the production of medically valuable cell populations like hematopoietic stem cells (HSCs) for therapeutic transplantation.

Though the first paper from Takahashi and Yamanaka established the feasibility of somatic cell reprogramming with a small set of defined factors, further refinements by his lab, as well as work by Hochedlinger and Jaenisch, yielded more faithfully reprogrammed cells and proved that iPSCs are indeed the functional equivalents of ESCs. Given the robustness of the original observation, numerous groups contributed to rapid improvements in reprogramming technology. In just over a year after the landmark reprogramming publication by Takahashi and Yamanaka, Yamanaka's (Takahashi et al., 2007) and Thomson's group (Yu et al., 2007), as well as my own (Park et al., 2008b), reported in late 2007 the successful reprogramming of human cells. In a blindingly quick period of time, the field turned its attention away from SCNT and converged on this new factor-based reprogramming technology to exploit its practical implications. We quickly applied reprogramming to patient-derived fibroblasts and reported the first large collection of disease-specific iPSC lines for conditions as diverse as Parkinson's, diabetes, and primary immune deficiency (Park et al., 2008a). Because of those early studies, hundreds of labs around the globe have embraced this facile technology to model and study the basic mechanisms of numerous human diseases (as reviewed in Robinton and Daley [2012]).

The Yamanaka experiments have ushered in an era of cellular alchemy. With cellular pathology apparent in vitro, numerous laboratories are interrogating disease mechanisms and testing chemicals and proteins as potential therapeutics to reverse the abnormal cell functions (Figure 1). Even more ambitious attempts are being launched to treat genetic diseases or acquired conditions of aging like macular degeneration with derivatives of iPSCs made from a patient's own cells. iPSCs can be entirely "self" or autologous cells, which theoretically escape the risk of immune rejection. Though this remains to be proven through clinical trials, this technology heralds an era during which any patient's cells represent the ingredients for tissue repair and regeneration. In a second major proof-of-principle experiment for the utility of customized pluripotent stem cells, Jaenisch and colleagues derived iPSCs from mice engineered to develop sickle cell anemia by virtue of expression of mutant human hemoglobin genes. They corrected the sickle globin gene defect, differentiated the cells in vitro into hematopoietic stem cells, and engrafted diseased recipients, which were subsequently cured of their condition (Hanna et al., 2007). This strategy represents a platform whereby dozens of genetic blood disorders could be treated by a common method and offers a glimpse of a future whereby

gene repair is coupled to cell replacement therapy for a diversity of conditions.

Yamanaka's bold experiment has further stimulated a whole host of new possibilities. If a small set of defined factors can reprogram a specialized cell back to pluripotency, what might stop a small set of factors from converting one specialized cell type into another? Indeed, dozens of papers now report using the Yamanaka method of screening small libraries of transcription factors for their capacity to transform one cell type into another, and the literature is buzzing with reports of cellular alchemy, whereby fibroblasts or other starting cell types are being converted into cardiomyocytes, neurons, hepatocytes, blood, and beyond (Vierbuchen and Wernig, 2011).

With such promise, key questions arise. When will medicines emerge from these Nobel-prize winning discoveries? The answer to this query is a difficult one, but given the rapid pace of adoption of iPSCs into the research armamentarium of so many labs, we might not be surprised if drug trials and the first cellular therapies emerge within the next 5 years. However, like any novel biotechnology, we must be realistic in concluding that two or three decades may be necessary before the applications of iPSCs in medicine are fully appreciated. In the meantime, intense research will continue.

A second and more controversial question asks whether there is an ongoing need for research on hESCs. Some political forces within the United States are using the occasion of the Nobel Prize to argue that research on human-embryo-derived stem cells is now superfluous. Such a perspective is founded on ideological and not scientific reasoning and is not supported by experts in iPSC derivation (Hyun et al., 2007). The discovery of murine iPSCs by Yamanaka was founded on decades of research that revealed the molecular machinery responsible for the pluripotent state of ESCs. The culture conditions for human and mouse ESCs are different, and knowledge of the specific conditions for growing hESCs was essential to the success of deriving human iPSCs. Indeed, in my own laboratory, our facility with deriving human iPSCs was due in large part to our experience deriving novel hESC lines. Without our expertise in identifying the morphology of hESCs and our facility with maintenance of healthy hESC cultures, we would not have been successful deriving human iPSCs. Though research on human iPSCs has exploded because of the ease of reprogramming and the power of its applications in medicine, there remain numerous reasons why research on hESCs must continue. There has been a decade more experience with hESCs than with human iPSCs, and two clinical trials of cell products of hESCs have already begun. We stand to learn invaluable lessons about the properties of all pluripotent stem cell products through these initial human clinical experiments. Moreover, many basic questions about the growth conditions of hESCs remain to be answered, especially given that mouse and human ESCs and iPSCs differ in fundamental properties. How the X chromosome functions in iPSCs and differentiated products that might come from iPSCs remains mysterious and will likely be understood best in direct comparison to the function of the X chromosome during the derivation of new hESC lines under new conditions.

Science progresses most efficiently when all possible avenues of exploration remain open. This year's Nobel Prize celebrates the creativity and brilliance that can be achieved when scientists of the highest caliber are free to ask bold questions.

SUPPLEMENTAL INFORMATION

Supplemental Information includes an annotated version of Takahashi and Yamanaka (2006) and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.11.016.

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